




Benchtop Isolation and Characterisation of Small Extracellular Vesicles from Human Mesenchymal Stem Cells

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Abstract

The objective of this study is to develop a simple protocol to isolate and characterise small extracellular vesicles (sEVs) from human umbilical cord-derived MSCs (hUC-MSCs). hUC-MSCs were characterised through analysis of morphology, immunophenotyping and multidifferentiation ability. sEVs were successfully isolated by ultrafiltration from the conditioned medium of hUC-MSCs. The sEVs' size distribution, intensity within a specific surface marker population were measured with zetasizer or nanoparticle tracking analysis. The expression of surface and internal markers of sEVs was also assessed by western blotting. Morphology of hUC-MSCs displayed as spindle-shaped, fibroblast-like adherent cells. Phenotypic analysis by flow cytometry revealed that hUC-MSCs expressed MSC surface marker, including CD90, CD73, CD105, CD44 and exhibited the capacity for osteogenic, adipogenic and chondrogenic differentiation. Populations of sEVs with CD9, CD63 and CD81 positive were detected with size distribution in the diameter of 63.2 to 162.5 nm. Typical sEVs biomarkers such as CD9, CD63, CD81, HSP70 and TSG101 were also detected with western blotting. Our study showed that sEVs from hUC-MSCs conditioned medium were successfully isolated and characterised. Downstream application of hUC-MSCs-sEVs will be further explored.

Keywords Mesenchymal stem cell · Small extracellular vesicle · Exosome · NTA · Regeneration

Introduction

Mesenchymal stem cell (MSC) is non-haematopoietic, multi-potent adult stem cell, capable of being isolated from multiple human tissues. Generally, MSCs can be isolated from Wharton's jelly, adipose tissue, periosteum, skeletal muscle,

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synovium, liver, spleen, thymus, placenta, brain, lung, palatine tonsils, peripheral blood and a few other tissues [1, 2]. They possess the ability to differentiate into mesodermal lineage such as osteocytes, adipocytes and chondrocytes. MSCs also have the ability to migrate to injured tissue and due to their differentiation capability into various cell lineages and through secretion of soluble molecules, they are able to regenerate injured tissues. Besides, MSCs have significant immunomodulatory and pro-angiogenic properties which allow MSCs to modulate immune responses [3]. The immunomodulatory effect in MSCs are widely mediated by differentiation of MSCs, paracrine signals, and several secreted molecules such as microvesicles [2].

Extracellular vesicle (EV) is defined by International Society for Extracellular Vesicles (ISEV) as the particle naturally secreted by the cell that are membrane-bounded by a lipid bilayer and doesn't undergo replication [4]. EV contains different types of membranous components in the 20–1000 nm diameter which are released by most of the cell types into the extracellular microenvironment [2, 5, 6]. EV consist of proteins, DNA and RNA species, lipid and metabolites which are found to be important mediators during intercellular communication for both short and longer-range signalling events [5, 7, 8]. It has been reported that the protein cargo EV carries are associated with normal physiologic and disease states, and the content can reflect biological event and disease progression, thus the biomarker potential of EVs is a subject of intense investigation. Cargos that EVs carry are also able to determine their mode of action in target cells via receptor-ligand interactions or transportation of cargo [8].

Small extracellular vesicles (sEVs) are generally represented as EV in the size of < 200 nm, which includes ectosomes and exosomes [4]. Exosome is a nanosized vesicle (diameter of 30 to 200 nm) which take parts in intercellular communication and transport proteins, lipids, and nucleic acid in between cells or across tissues [9]. Exosome risen as a novel cell-less regenerative medicine due to its unique properties [10]. The biological factors carried by exosomes are suggested to contribute in the protection, regeneration and angiogenesis of damaged tissues [11]. Multiple studies demonstrated the regenerative effect of MSCs-derived sEVs. For instances, MSCs-derived sEVs protect the damaged kidney, stimulate hepatic regeneration and cardiac repair [12–14]. In this study, we developed a simple protocol to isolate and characterise sEVs from human umbilical cord mesenchymal stem cell (hUC-MS-C) with accordance to ISEV guideline.

Materials and Methods

Chemicals

Bovine serum albumin (BSA), bromophenol blue, Chemi-Lumi One L, dimethyl sulfoxide (DMSO), paraformaldehyde, penicillin–streptomycin (Pen-Strep), low glucose (5.6 mmol/L)-Dulbecco's modified Eagle's medium (LG-DMEM), phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, Protein Assay Bicinchoninate Kit (06,385–00), sample buffer solution with 2-ME (2x) for SDS-PAGE, sodium chloride (NaCl), sodium lauryl sulfate (SLS), tetramethylethylenediamine (TEMED), 40% acrylamide and 95% ethanol were purchased from Nacalai Tesque (Kyoto, Japan). CryoStor® Freezing Media was purchased from Sigma-Aldrich (St Louis, MO, USA). Glycerol was purchased from Merck (Darmstadt, Germany). Primary mouse antibodies anti-CD 9 (C-4: sc-13118), anti-CD 63 (MX-49.129.5: sc-5275), anti-CD 81 (B-11: sc-166029), anti-HSP 70 (3A3: sc-32239), anti-GRP 94 (H-10: sc-393402), anti-TSG 101 (C-2: sc-7964), anti-ALB (F-10: sc-271605) and secondary antibodies horseradish peroxidase-conjugated mouse IgG kappa binding protein (m-IgGk BP-HRP) (sc-516102) were purchased from Santa Cruz Biotechnology (CA, USA). BD Stemflow™ Human MSC Analysis Kit (562,245), PerCP-Cy™5.5 Mouse Anti-Human CD63 (565,426), PerCP-Cy™5.5 Mouse anti-Human CD9 (561,329) and PE Mouse Anti-Human CD81 (561,957) were purchased from BD (Singapore). Foetal bovine serum (FBS) was purchased from Tico Europe (Amstelveen, Netherlands). KnockOut™ DMEM (KO-DMEM), GlutaMAX™ supplement, phosphate-buffered saline (PBS), StemPro™ Adipogenesis Differentiation Kit (A1007001), StemPro™ Chondrogenesis Differentiation Kit (A1007101) and StemPro™ Osteogenesis Differentiation Kit (A1007201) were purchased from Gibco™ (New York). Accutase™ was purchased from STEMCELL™ Technologies (Vancouver, Canada). Cell lysis buffer was purchased from Invitrogen (Carlsbad, California). Glycine and tris-base were purchased from 1st Base (Singapore). Absolute Methanol and ammonium persulfate (APS) were purchased from Chemiz (Shah Alam, Malaysia). ExcelBand™ Enhanced 3-color High Range Protein Marker (PM2610) was purchased from SMOBIO (Taiwan). Tween 20 (50%) was purchased from GeneTex (San Antonio, Texas, USA). Extra thick blotting paper and polyvinylidene fluoride (PVDF) membrane with 0.45 mm pore size were purchased from ATTO (Tokyo, Japan). Centricon® Plus-70 (100 kDa NMWL) was purchased from Millipore™.

Human Umbilical Cord Mesenchymal Stem Cells and Culture

The human umbilical cord mesenchymal stem cells (hUC-MSCs) was purchased from Tissue Engineering Centre, UKM Medical Centre. The cells were grown in KO-DMEM, supplied with 10% FBS, 1% Pen-Strep, 1% GlutaMAX™ supplement and incubated at 37 °C in 5% CO₂ supplement. The cell culture medium was replaced every 3 days. The hUC-MSCs cultured from the early passages (passage 3–5) were used for experiment.

Characterisation of hUC-MSCs

Morphological Study

The hUC-MSCs morphology was observed under Nikon Eclipse Ti™ inverted light microscope every 3 days at magnification of 100× and 200×. Images were captured at the same spot.

Flow Cytometry Analysis

The evaluation of hUC-MSCs' cell surface markers was carried out according to the instruction in BD Stemflow™ Human MSC Analysis Kit. Approximately 5 million of hUC-MSCs was required to carry out the analysis. The cells were detached from the cell culture flask using Accutase™ cell detachment. The detached cells were washed once, centrifuged (1000 rpm, 5 min) and suspended in PBS with cell concentration (1×10^6 cells/mL). The cell suspension was then transferred into 10 tubes of 1.5 mL of microcentrifuge

tubes with cell number of 5×10^5 in each tube and added with antibodies as specified in the Table 1. The tubes were then incubated in dark for 30 min at room temperature. After incubation, the cells were washed once with PBS, centrifuged (1000 rpm, 5 min) and suspended in 300 μL of PBS. The cells were analysed using flow BD Accuri™ C6 cytometer.

Assessment of Multi-differentiation Potential

Gibco™ StemPro™ Adipogenesis Differentiation Kit, StemPro™ Chondrogenesis Differentiation Kit, and StemPro™ Osteogenesis Differentiation Kit were used to differentiate the hUC-MSCs into adipocytes, chondrocytes and osteocytes, respectively, according to the manufacturer's protocol. When the confluency of hUC-MSCs reached 70%, the cells were detached and replated in 6-wells plate at cell number of 2×10^5 for each well. After overnight incubation in LG-DMEM with 10% FBS and 1% Pen-Strep, the medium were replaced with Complete Adipogenesis-, Chondrogenesis-, and Osteogenesis-differentiation medium in duplicates. The cell culture media was replaced accordingly every 3 days. For each lineage to differentiate from hUC-MSCs, it requires a specific cultivation period: adipogenic, chondrogenesis and osteogenic cultures was 7–14 days, > 14 days and > 21 days, respectively. To analyse the differentiation of hUC-MSCs, different staining procedures were performed to validate the occurrence of differentiation. Briefly, the cells were fixed before proceeding to staining. Thus, at the end of the culture period, the media was removed from the wells and rinsed with PBS once. The cells were fixed in 4% paraformaldehyde/PBS at room temperature for 30 min and rinsed twice

Table 1 Antibodies staining of human umbilical cord-derived mesenchymal stem cells for flow cytometry analysis

Tube number	Description	Antibodies
1	Cell alone	–
2	Compensation Control	CD90 FITC
3	Compensation Control	CD44 PE
4	Compensation Control	CD105 PerCP-Cy5.5
5	Compensation Control	CD73 APC
6	Positive Marker Cocktail	CD90 FITC CD105 PerCP-Cy5.5 CD73 APC
7	Negative Marker Cocktail	CD34 PE CD11b PE CD19 PE CD45 PE HLA-DR PE
8	Positive Isotype Control Cocktail	mIgG1, κ FITC mIgG1, κ PerCP-Cy5.5 Cocktail mIgG1, κ APC
9	Negative Isotype Control Cocktail	mIgG1, κ PE mIgG2a, κ PE
10	Isotype Control (for CD44 drop in marker)	mIgG2b, κ

with distilled water at the end the fixation. For adipocytes, the cells were stained with Oil Red O solution at room temperature for 30 min. After incubation, the wells were rinsed with distilled water 3 times and observed under light microscope. For chondrocytes, cells were stained with 1% Alcian Blue solution in 0.1 N HCl at room temperature for 30 min. After incubation, the wells were rinsed 3 times with 0.1 N HCl and distilled water was added to neutralize the acidity. The cells were then observed under light microscope to identify presence of blue staining. Lastly, for osteocytes, cells were stained with 2% Alizarin Red S solution at room temperature for 2–3 min. The wells were then rinsed 3 times with distilled water and observed under microscope for red staining.

Small Extracellular Vesicles Isolation from hUC-MSCs

When the hUC-MSCs have reached 70–80% confluency, the culture medium was replaced with fresh KO-DMEM supplemented with 1% GlutaMAX™ and 1% Pen-Strep [Conditioned medium (CM)]. After 48 h, the CM was collected from the flask. To minimize the variables between each batch of sample, only the CM harvested from hUC-MSCs at passage 5 was used for sEVs isolation. The CM was filtered through 0.22 µm filter to remove death cells, debris and any other particles larger than 220 nm. Millipore™ Centricon® Plus-70 (100 kDa NMWL) was then utilised to isolate sEVs. Centricon® Plus-70 was filled with filtered CM and centrifuged at 3500 × *g* for 20 min at 4 °C. The solution in filtrate collection cup was discarded. The sample filter cup with concentrate collection cup was reverse centrifuged at 1000 × *g* for 2 min at 4 °C to obtain the sEVs. To further purify the sEVs, sEVs were diluted with PBS (70X dilution) and recentrifuged at 3500 × *g* for 20 min at 4 °C. After discarding the solution in filtrate collection cup, sample filter cup with concentrate collection cup was reverse centrifuged at 1000 × *g* for 2 min at 4 °C to collect the cleaned sEVs. The samples were then transferred to new tubes and stored in – 80 °C for further analysis.

Characterisation of hUC-MSC-Derived sEVs

Western Blotting Analysis

hUC-MSC and sEVs were lysed with ice-cold radio immunoprecipitation assay (RIPA) lysis buffer, incubated for 30 min at 4 °C and centrifuged at 200 × *g* for 5 min at 4 °C. The supernatants were transferred to a new 1.5 mL centrifugal tube and stored at – 80 °C until further use. After protein quantification with Protein Assay Bicinchoninate Kit, the samples were then separated by gel electrophoresis and transferred to PVDF membrane. After blocking with 3% BSA/TBS-T for 1 h on shaker at room temperature,

the samples were incubated with primary antibodies: mouse anti-CD 9 monoclonal antibody (1:500), mouse anti-CD 63 monoclonal antibody (1:500), mouse anti-CD 81 monoclonal antibody (1:500), mouse anti-HSP 70 monoclonal antibody (1:500), mouse anti-GRP 94 monoclonal antibody (1:500), mouse anti-TSG 101 monoclonal antibody (1:500) and mouse anti-ALB (1:500) under 4 °C for overnight with constant shaking. On the next day, the PVDF membrane was washed 5 times with TBS-T and further incubated with secondary antibody: Horseradish Peroxidase-conjugated mouse IgG kappa binding protein (m-IgGκ BP-HRP) (1:5000) for an hour with agitation at room temperature. After washing 5 times with TBS-T, the membrane was visualized using UVP™ (Ultra Vision Product) CCD imager with Chemi Lumi-One L Detection Reagent. The expression level of the proteins was analysed using ImageJ software.

Dynamic Light Scattering Measurement

The particle size of sEVs was measured using a Zetasizer Nano ZS (Malvern Panalytical, UK). The sEV samples were first diluted with filtered PBS at a dilution factor of 5:800. Next, 1 mL of diluted sEV samples were loaded into a folded capillary cell. The folded capillary cell was pre-rinsed with filtered ultrapure water before introducing the sample. The temperature of the cuvette holder was equilibrated to 25 °C before initiating analysis using the Zetasizer Nano software. The samples are analysed in triplicates under the normal resolution settings.

Nanoparticle Tracking Analysis

sEVs' size distribution curves and concentration measurements was carried out by nanoparticle tracking analysis (NTA) with Particle Metrix ZetaView®. To conduct the assay, the samples in liquid form was loaded into a sample chamber. Particles in the path of the beam scatter the laser light was then collected by the 20 × microscope objective and viewed with a digital camera. The Brownian motion of multiple particles was analysed individually and simultaneously (particle-by-particle). The particles count number was maintained at 10–100 by performing dilution to the sample. The hydrodynamic diameters of sEVs was calculated using the Stokes Einstein equation. sEVs were then further stained with anti-CD9-PerCP-Cy™5.5, anti-CD63-PerCP-Cy™5.5 and anti-CD81-PE to further visualise and calculate the sEVs based on their specific markers. The resulting videos were analysed with the ZetaView® software 8.05.10 (Particle Metrix, Germany).

Statistical Analysis

Statistical analysis for the quantitative data and results obtained was performed by IBM SPSS® Statistics 20 software to determine the significance of results. All the data and results collected were analysed and interpreted as mean \pm S.D. Results were analysed using One-Way ANOVA and post hoc test. Statistical significance was considered at $p < 0.05$.

Results

hUC-MSCs Expressed the Typical MSC Surface Markers

The primary and passaged hUC-MSCs consistently grow in monolayer and displayed spindle shape (fibroblast-like) (Fig. 1). We have also cultured the cells up to passage 10 and the morphology of the cells remained as described

earlier. No alteration in morphology was observed (Data not shown). To further validate the hUC-MSCs, the cells were analysed for mesenchymal cell surface markers and haematopoietic markers by flow cytometry. As shown in Fig. 2, hUC-MSCs were positive for CD73, CD90, CD105 and CD44 with $98.83 \pm 0.74\%$, $98.8 \pm 1.25\%$, $97.73 \pm 0.95\%$ and $98.93 \pm 0.54\%$, respectively but negative for CD45, CD34, CD11b, CD19, HLA-DR PE (negative cocktail, $1.48 \pm 0.93\%$).

hUC-MSCs Differentiated into Adipocytes, Chondrocytes and Osteocytes

The differentiation potential of hUC-MSCs was assessed through adipogenic, chondrogenic and osteogenic differentiation. Lipid droplets were observed in the cytoplasm of the cells after 2 weeks of culture in complete adipogenesis differentiation medium. This was evidenced by the red spots in the cells (Fig. 3A). Similarly, chondrogenic differentiation was assessed with Alcian Blue staining which showed

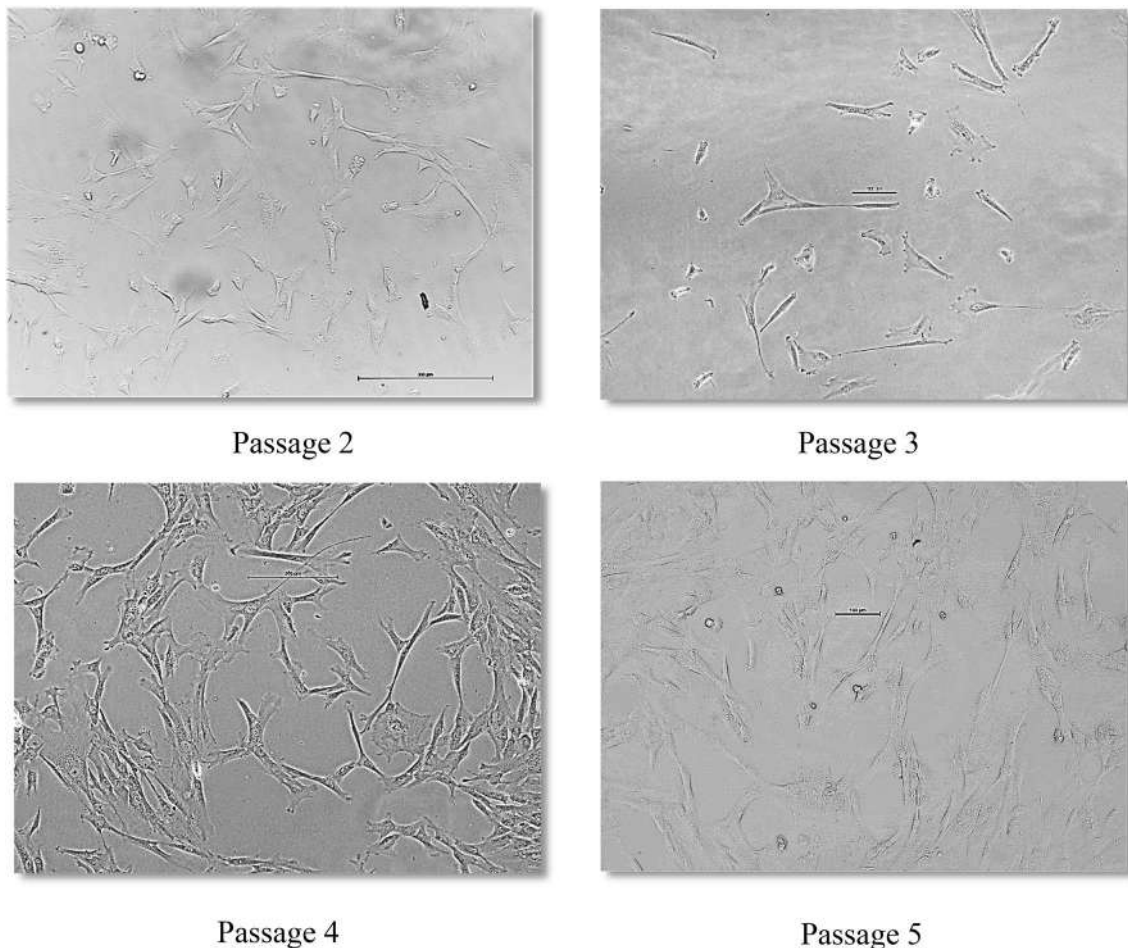


Fig. 1 The morphological characteristic of cultured human umbilical cord-derived mesenchymal stem cells (hUC-MSCs). hUC-MSCs were observed as spindle-shaped fibroblast-like cells which grows adherently throughout the passage 2 to passage 5 (under magnification of 20 \times)

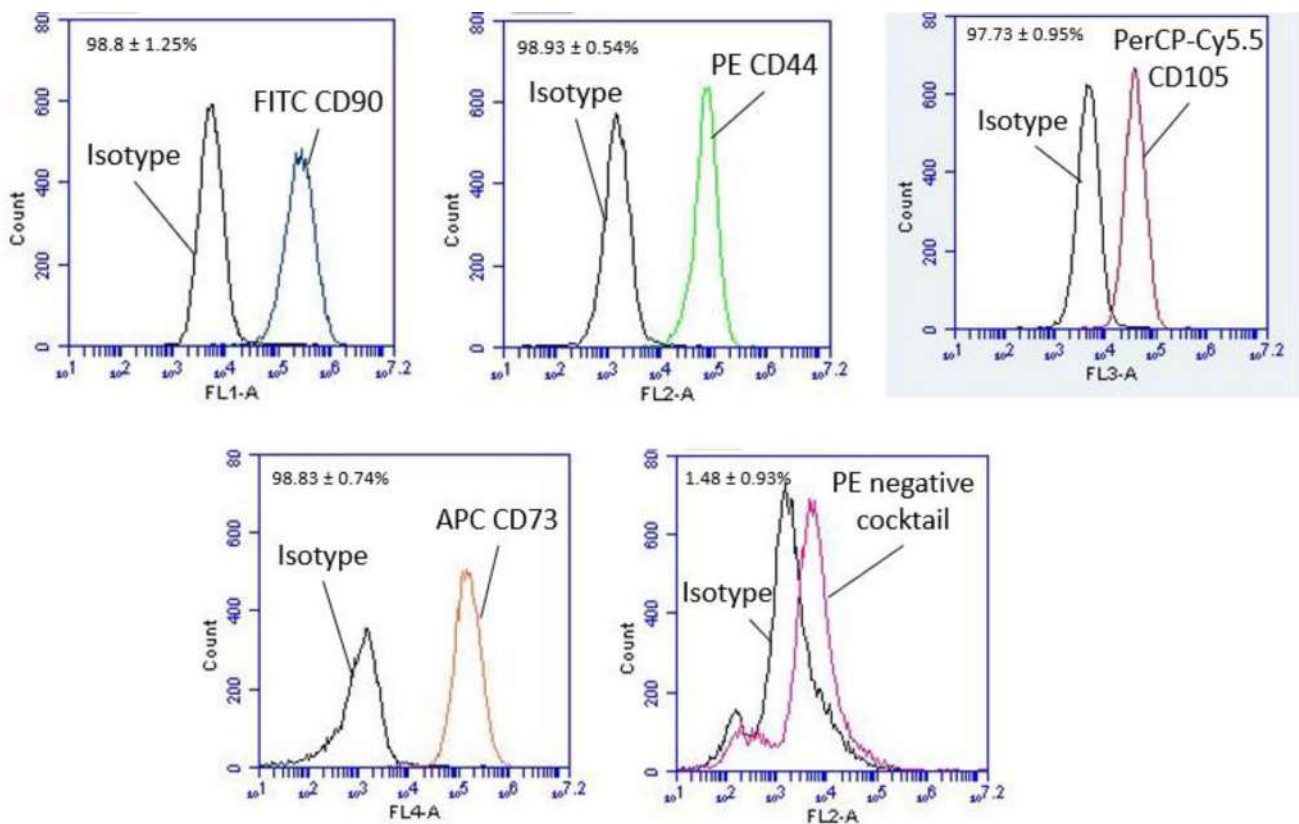


Fig. 2 The phenotypical characteristic of cultured hUC-MSCs. The flow cytometry analysis shows presence of CD90, CD44, CD105 and CD73, and absence of CD34, CD11b, CD19, CD45 and HLA-DR (Cocktail) in P5 hUC-MSCs

proteoglycan expression (blue circle) by the cells (Fig. 3C). After induction in complete osteogenesis differentiation medium for 3 weeks, calcium phosphate presence in extracellular matrix of the cells were assessed by Alizarin Red staining (the darker orange spots shown in Fig. 3E). Besides that, spontaneous differentiation was not observed in the control cells cultured with complete growth KO-DMEM medium (Fig. 3B, D, F).

sEVs were Successfully Isolated and Characterised

The properties of sEVs isolated from hUC-MSCs were characterised based on criteria set by ISEV, including the particle size range, the detection of specific internal and external biomarkers. We first measured the sEVs particle size using Zetasizer. Result showed that the average size range of sEVs was 128.3 nm with PDI value 0.312 (Fig. 4). Particle size distribution (PSD) by intensity showed a single peak ranging from 90 to 300 nm (Fig. 4B). It is worth noting that no large particles were detected in PSD by intensity, indicating the effectiveness of the sEVs isolation process. The proteins of sEVs were then separated by gel electrophoresis and stained with coomassie brilliant blue. The major proteins present in

sEVs were above 45 kDa with a prominent protein band at approximately 55 kDa (Fig. 5).

To further verify the external and internal biomarkers of the sEVs samples, western blot analysis was conducted. sEVs expressed both external (CD63, CD9, CD81) and internal biomarkers (HSP70 and TSG101). Although hUC-MSC cell lysate expressed CD63, however, other sEVs markers (CD9, CD81, HSP70 and TSG101) were not detected in the cell lysate. It should also be noted that GRP94 expression was expressed in hUC-MSC cell lysate but absent in sEVs (Fig. 6).

The Number of sEVs was Quantified with Nanoparticle Tracking Analysis

The particle size of sEVs was also measured by nanoparticle tracking analysis (NTA). The total number of particles (hybrid) was 5.4×10^{11} particles/mL. The number of sEVs that expressed CD63, CD81 and CD9 was 1.4×10^{10} particles/mL, 2.1×10^{10} particles/mL and 2.4×10^{10} particles/mL, respectively (Fig. 7). The size distribution for all these analysed samples fall within the expected size range for sEVs (30–200 nm). The modes of all the sEVs samples are approximately 120 nm.

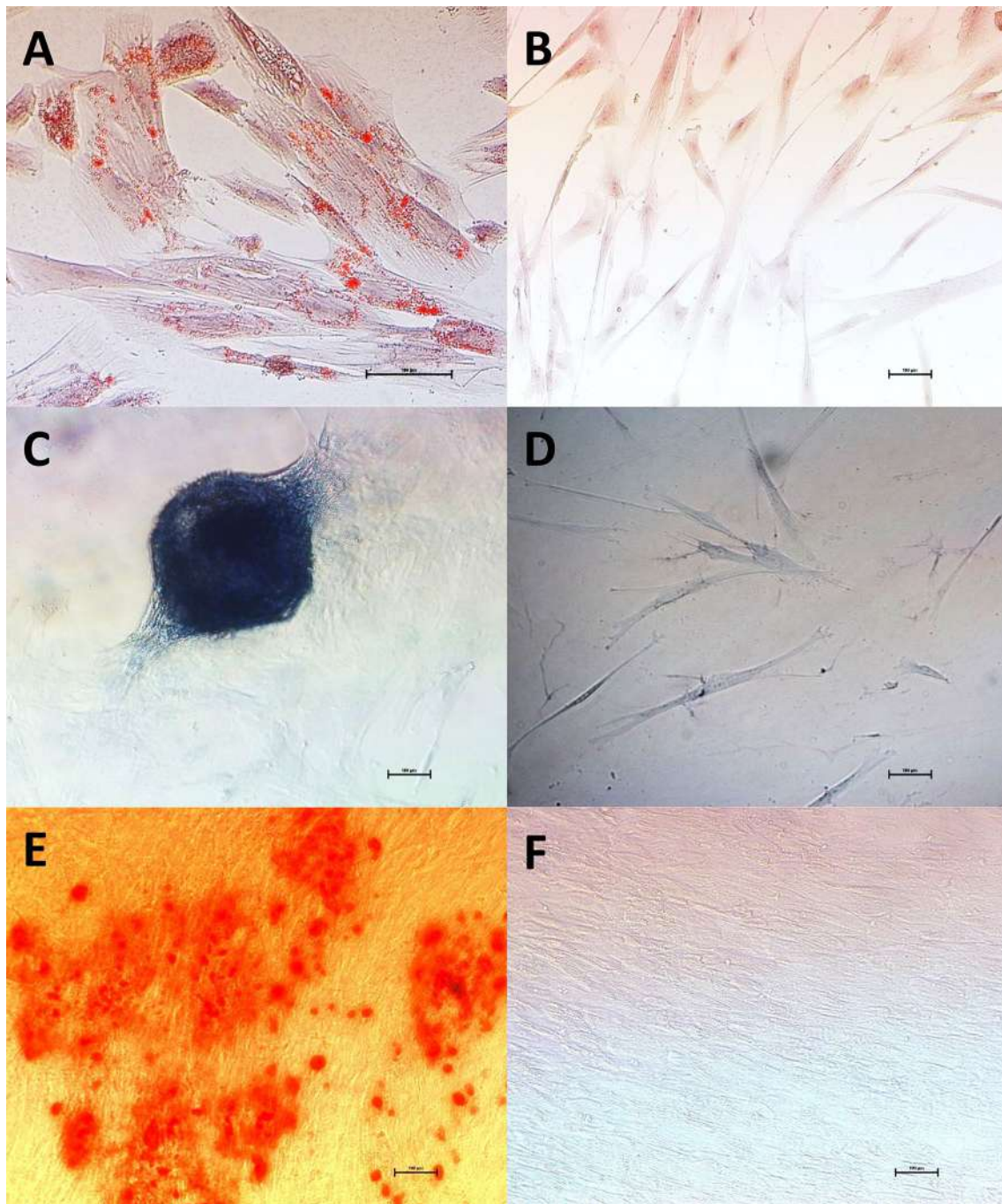


Fig. 3 The multilineage differentiation potential of cultured hUC-MSCs. The P5 hUCMSCs were cultured in adipogenic, chondrogenic and osteogenic medium for 2–3 weeks. **A:** Lipid droplets observed in cytoplasm after 2 weeks induction through Oil-Red-O staining. **C:** Chondrogenic differentiation was assessed with Alcian Blue staining

which shows proteoglycan expression by the cells. **E:** Calcium phosphate presence in extracellular matrix of the cells were assessed by Alizarin Red staining. The control groups (**B, D, F**) were cultured in completed KO-DMEM medium simultaneously and no spontaneous differentiation observed (under magnification 20 \times)

Discussion

Amongst the cell types that are known to produce sEVs, MSC is an ideal candidate for mass production of sEVs for drug delivery and regenerative medicine [15]. Various

disease models have been studied based on MSCs-derived sEVs and results demonstrated that MSCs and MSCs-derived sEVs have similar function in organisms, including tissue repair, anti-inflammation and immune system modulation [2]. The intravenous injection of hUC-MSCs-derived

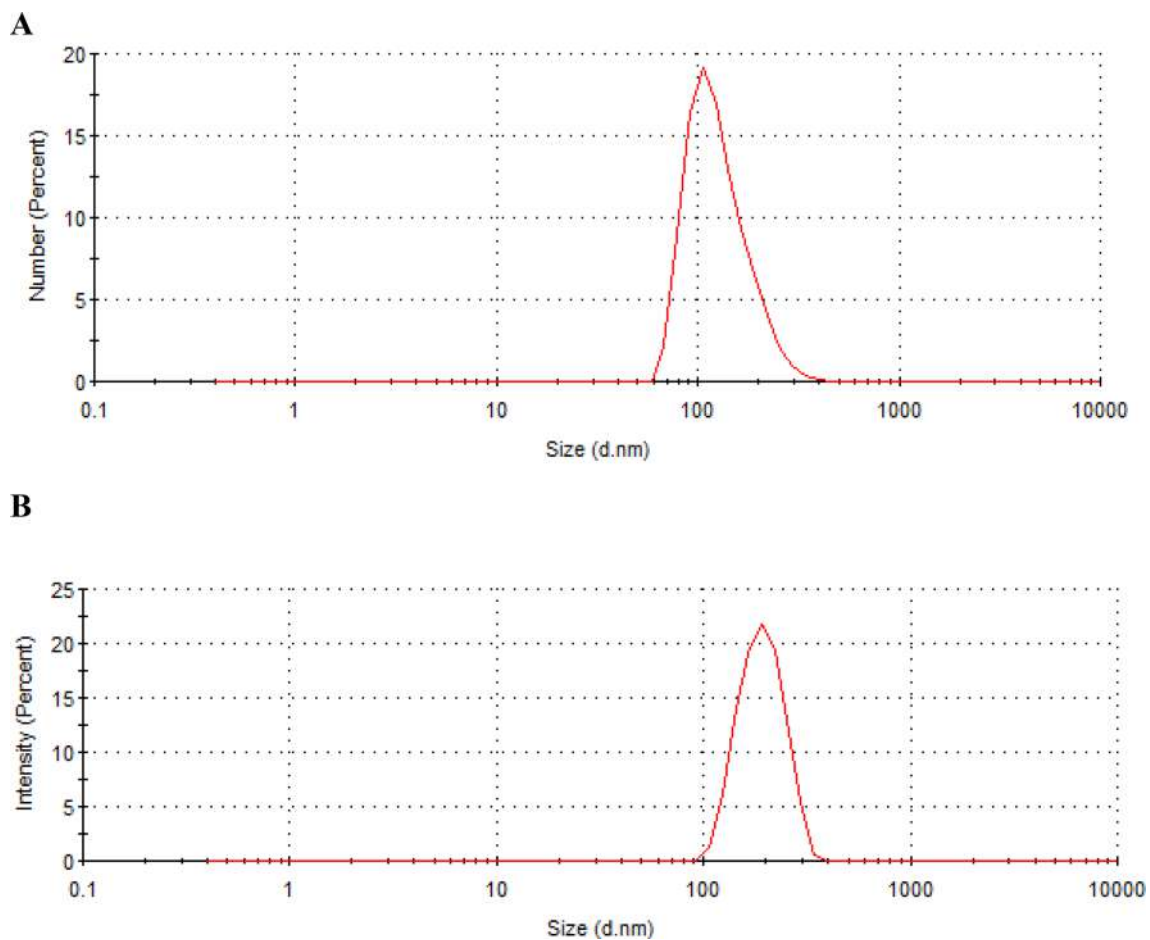


Fig. 4 Particle size distribution of hUC-sEVs. These figures are representation of DLC analysis by **(A)** number and **(B)** intensity in triplicate

sEVs in animal models revealed a supportive effects on weight loss and had no harmful effects on renal or liver function [2].

To study the sEVs and their biological functions in human health, the isolation and quantification of sEVs have become a major initiative in both basic research and clinical applications. Currently, the sEVs isolation techniques including differential ultracentrifugation, ultrafiltration, polymer-based precipitation, immunoaffinity capture, microfluidics-based techniques etc. Each technique has innate advantages and disadvantages in sEVs isolation [7, 16, 17]. However, none of these single optimal isolation technique able to achieve the high recovery rate and high specificity feature, thus researchers have to choose the isolation method based on the downstream applications to compromise in between recovery and specificity [4]. In the field of stem cells research, ultrafiltration appears to be one of the most favourable techniques in MSC-sEVs isolation as the products are free from any chemicals, high yield and scalable. A technical research paper by Lobb et al. (2015) compared the yield of sEVs isolated from lung cancer cells with various methods including

ultrafiltration, ultracentrifuge and commercial kits. Results showed that ultrafiltration gave the highest sEVs yield in comparison to other methods [18]. This information can be translated that ultrafiltration is the best method that will give the highest stem cells' sEVs yield during manufacturing process. To the best of our knowledge, there is no technical paper to compare the yield of stem cells sEVs harvested with different methods. A proper designed experiment is needed to maximise the stem cells' sEVs yield with minimum cost of production.

In this study, we have successfully characterised hUC-MSCs based on the minimal criteria for defining multipotent mesenchymal stromal cells as ruled out by The International Society for Cellular Therapy and developed a simple bench-top protocol to isolate sEVs (Fig. 8)[19]. Our hUC-MSCs displayed spindle shape (Fig. 1), expressed all the MSC markers (Fig. 2) and able to be differentiated into adipocytes, chondrocytes and osteocytes (Fig. 3). For characterisation of sEVs, various techniques were also used including western blot and NTA [4]. The size of the isolated sEVs was less than < 200 nm which was within the expected "sEV" size

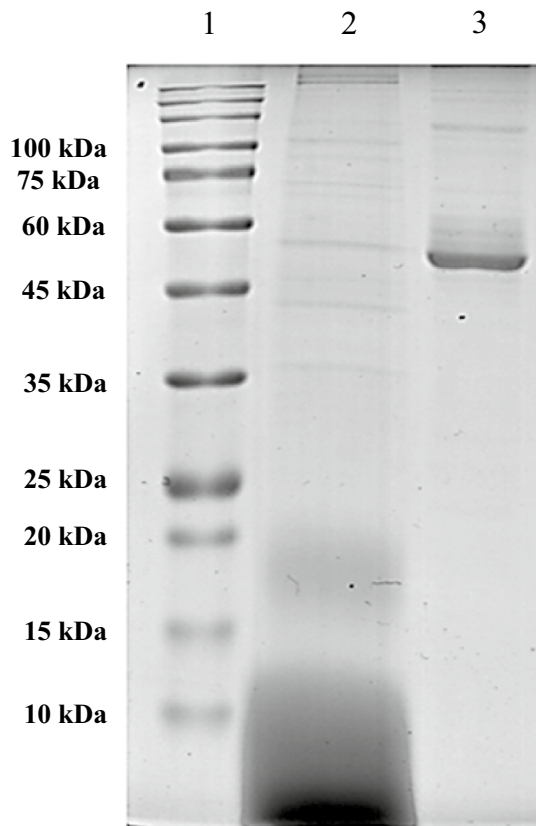


Fig. 5 The protein profile of sEVs. Distant profile of protein was observed between hUCMSC cell lysate and sEVs from ultrafiltration. Lane 1: Protein marker; Lane 2: hUC-MSC cell lysate; Lane 3: sEVs

(Fig. 4). Indeed, our sEVs expressed both sEVs external markers (CD63, CD81, CD9) and internal markers (TSG101 and HSP70) (Fig. 6 and Fig. 7). The absence of GRP94 marker in our sEVs also revealed that the isolation was clean and free from any cell debris. It should be noted that the removal of contamination of non-exosomal particles, such as apoptotic bodies, small apoptotic vesicles, exomeres, and lipoproteins is important as these contaminants are able to cause false conclusion regarding the biological activities of obtained sEVs. As the serum are likely to contain EVs, thus the EV-free serum or basal media is recommended to avoid external “EV-contaminants”. As current studies mostly harvest their sEVs from the cell cultures, the utilisation of serum-free medium or exosome-free serum is an important criteria to avoid the contamination [20].

In the detection of sEVs markers with immunoblotting, there is a trick that need to be aware by the researchers. We initially included reducing agent 2-mercaptoethanol into our sEVs for immunoblotting. Nevertheless, the expression of external markers (CD63, CD9 and CD81) was hardly detected (data no shown). In view of the reducing property of 2-mercaptoethanol that might have denatured

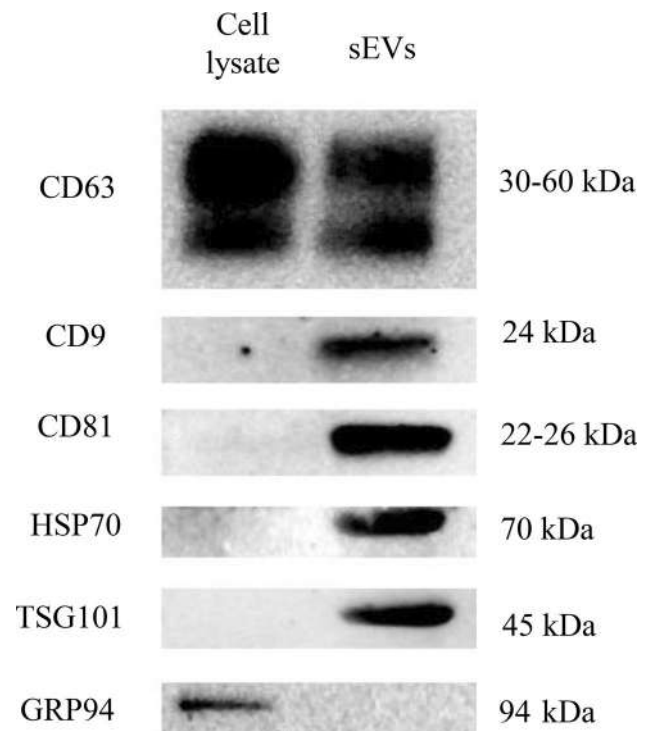
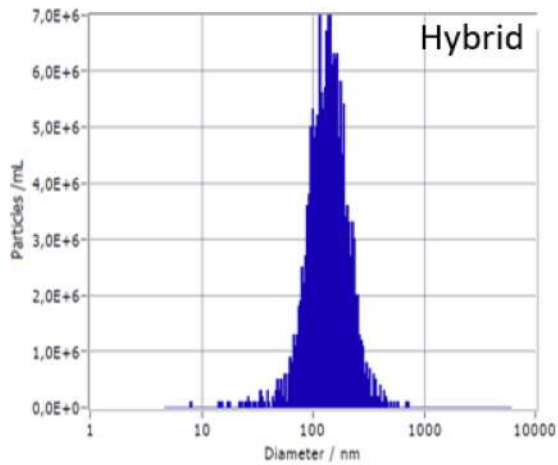


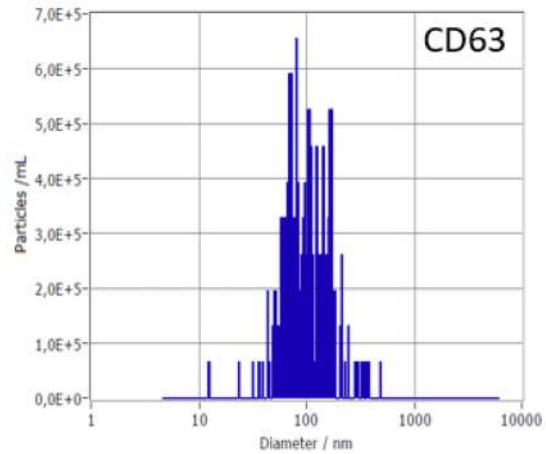
Fig. 6 The biomarkers expression of sEVs. Western blot was carried out to further confirm the expression of sEVs biomarkers. The sEV samples expressed the typical markers of sEVs including CD63, CD9, CD81, HSP70, TSG101 and absent in GRP94. The image was a representative image from three independent tests

the protein structure of sEVs external markers, 2-mercaptoethanol was then removed from the samples. Finally, sEVs external markers were easily detected and clearly seen [21].

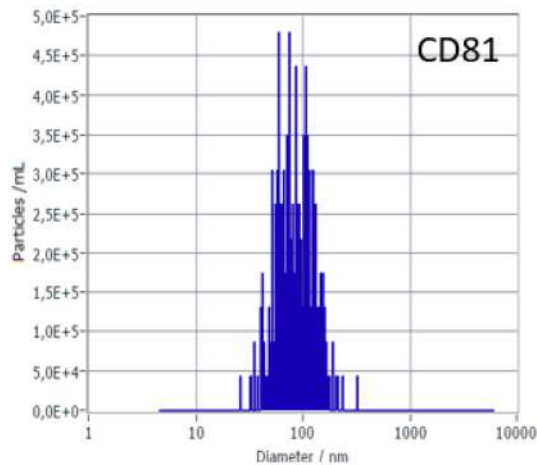
For future clinical application of MSC-derived sEVs, there is a debatable question that need to be addressed by the researcher: The treatment plan should be based on number of particle of sEVs or protein concentration? Which one will be more accurate or reliable? Protein concentration has long been used as the treatment guide in regenerative medicine in preclinical stage [22–26] and recently studies have been reported to employ “number of particles of sEVs” as treatment guide [27–31]. These two different treatment guides seem effectively repaired the damaged tissues or promoted tissue regeneration as evidenced by the above-mentioned studies. Although NTA is available for the quantification of sEVs particle, nevertheless, this instrument may not be available in many laboratories. To the best of our knowledge, no study has been conducted to evaluate which treatment guide (number of particle of sEVs vs sEVs concentration) is more effective in regenerative medicine. It is therefore recommended that a study should be properly designed with stringent isolation and characterisation protocol to obtain



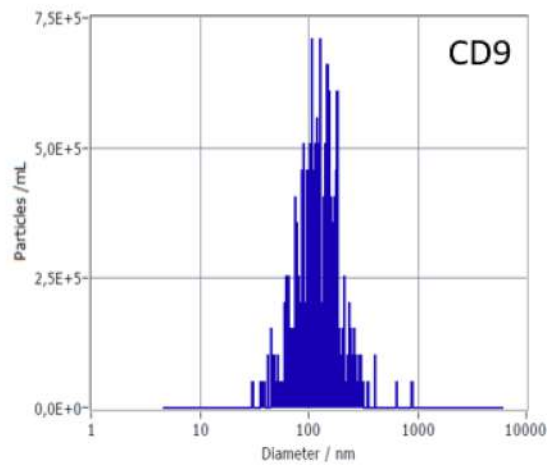
Total number: 5.4×10^{11} particles/mL
Mean size: 148.9 nm



Total number: 1.4×10^{10} particles/mL
Mean size: 109.2 nm



Total number: 2.1×10^{10} particles/mL
Mean size: 135.4 nm



Total number: 2.4×10^{10} particles/mL
Mean size: 114.7 nm

Fig. 7 The size distribution of sEVs samples with corresponding surface markers stained. The sEV samples were incubated with 3 different sEVs specific biomarkers and visualised through nanoparticle

tracking analysis. Each of the biomarker-sEVs showed a significant peak around size of 110–140 nm in diameter. These figures are representation of NTA in triplicate

cleaned and pure sEVs to ensure the effectiveness of sEVs in regenerative medicine.

Conclusion

hUC-MSCs were successfully characterised in which the cells displayed as spindle-shaped, fibroblast-like adherent cells, expressed typical MSC surface marker and exhibited the capacity for osteogenic, adipogenic and chondrogenic differentiation. sEVs were also successfully

isolated with ultrafiltration method and well characterised in which the sEVs were CD9, CD63, CD81, HSP70 and TSG101 positive with size distribution in the diameter of 63.2–162.5 nm. Downstream application of hUC-MSCs-sEVs will be further explored in the near future.

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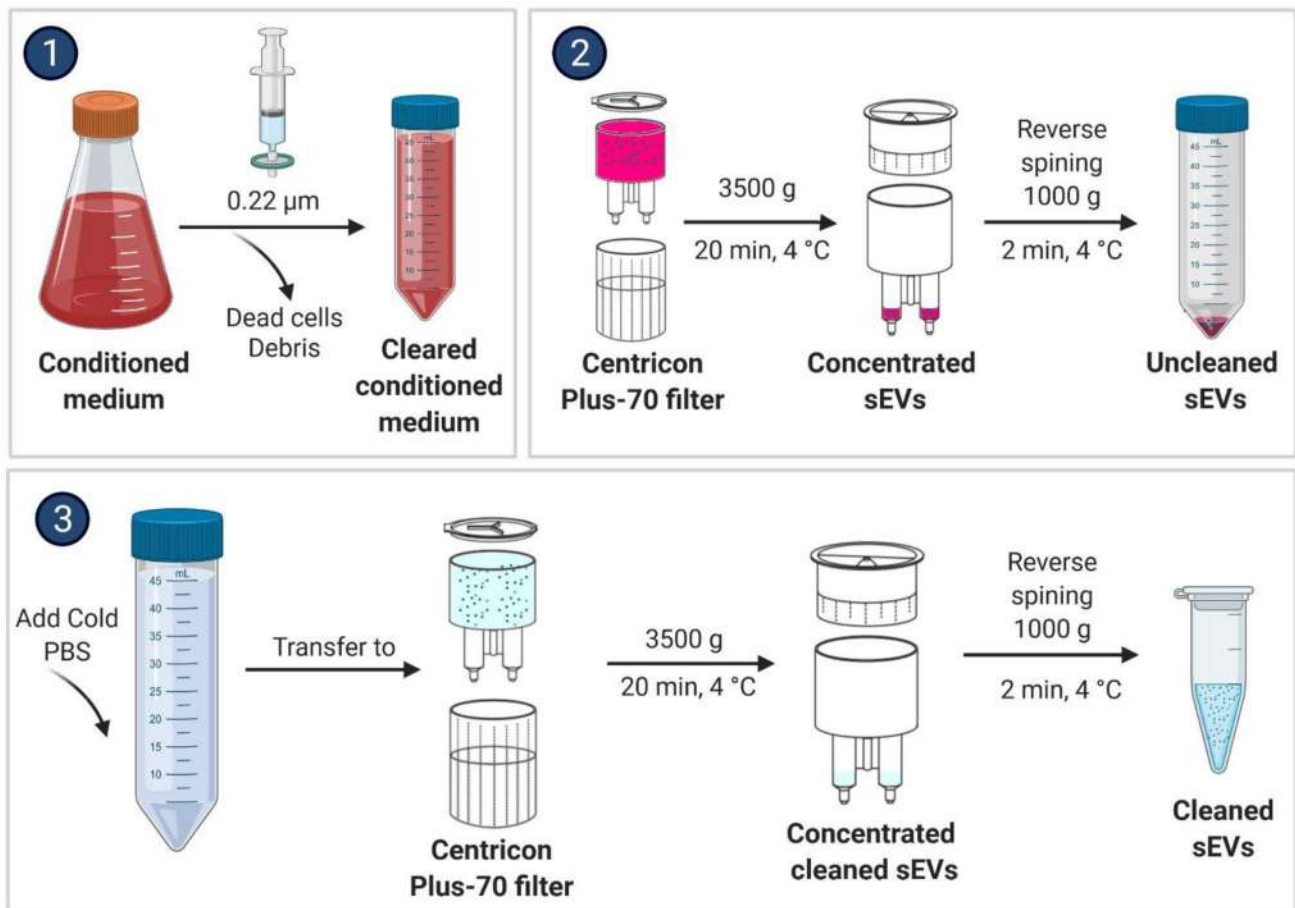


Fig. 8 Benchtop filtration protocol for the isolation of sEVs from mesenchymal stem cell conditioned medium. In Step 1, cells and cell debris are removed via microfiltration. sEVs and free protein can pass through 0.22 μm filter. In Step 2, free proteins are removed by ultra-

filtration and sEVs are concentrated. In Step 3, sEVs are washed once with PBS and reconcentrated to obtain cleaned sEVs (Created with BioRender.com)

Authors' Contribution JBF, QHDL and YST contributed to the design of the study and interpretation of data. KLT and WCC performed most of the data collection. CWH and PLS assisted in the data collection from Zeta Sizer and NTA. This manuscript is compiled by KLT, JBF and critically revised by YST, QHDL and CWH.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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